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Monoclonal Antibodies Directed Against a Cell Surface-Exposed Outer Membrane Protein of *Haemophilus influenzae* Type b

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Monoclonal antibodies directed against several different *Haemophilus influenzae* type b outer membrane proteins with apparent molecular weights of 45,000, 39,000, and 37,000 were identified by a radioimmunoprecipitation method. Five monoclonal antibodies, including both immunoglobulin G and M isotypes, were specific for the same *H. influenzae* type b major outer membrane protein (39,000 molecular weight). One of these immunoglobulin G monoclonal antibodies (6A2) was shown to be directed against a cell surface-exposed antigenic determinant of the 39,000-molecular-weight protein, whereas the other monoclonal antibodies directed against this same protein were apparently specific for antigenic determinants not exposed on the *H. Influenzae* type b cell surface. The cell surface-exposed protein antigenic determinant recognized by monoclonal antibody 6A2 was not unique to the *H. influenzae* type b strain used as the source of outer membrane vesicles for generating immune spleen cells, but was found in a majority of independently isolated strains of *H. influenzae* type b. These data indicate that there is antigenic cross-reactivity among *H. influenzae* type b strains with regard to cell surface-exposed proteins.

Haemophilus influenzae type b is the most important cause of endemic bacterial meningitis in infants (3), and the failure of the purified *H. influenzae* type b capsular antigen (phosphoribosylribitol phosphate) to induce the synthesis of protective antibodies in infants less than 14 months of age has necessitated the identification of alternative *H. influenzae* type b vaccinogens (6, 19). That antibodies directed against noncapsular somatic antigens can protect against experimental disease caused by encapsulated bacterial pathogens has been established by studies with such diverse organisms as *Neisseria meningitidis* (5) and *Streptococcus pneumoniae* (2). Similarly, antibodies directed against noncapsular cell surface antigens of *H. influenzae* type b have been shown to protect infant rats against experimental *H. influenzae* type b disease (7, 22). Moreover, young rats convalescing from systemic *H. influenzae* type b disease induced in infancy have been shown to mount a protective antibody response directed against these somatic noncapsular antigens (7, 22). However, the identity of the *H. influenzae* type b cell surface immunogens against which these protective antibodies were directed has not yet been established.

The two classes of *H. influenzae* type b cell surface antigens against which protective antibodies might be directed are the lipopolysaccharide and proteins present in the outer membrane of this pathogen. The inherent toxicity of most bacterial lipopolysaccharide molecules weighs against the ready utilization of this moiety as an *H. influenzae* type b vaccinogen. In contrast, proteinaceous vaccines such as the diphtheria-pertussis-tetanus vaccine are relatively nontoxic and immunogenic in infants (28); accordingly, there has arisen considerable interest in the possible use of outer membrane proteins of *H. influenzae* type b as vaccinogens (6, 10). That *H. influenzae* type b outer membrane proteins are immunogenic in very young mammals has been established by a previous study from this laboratory, in which young rats convalescing from systemic *H. influenzae* type b disease were shown to mount an antibody response directed against several different *H. influenzae* type b outer membrane proteins (10). More importantly, we have recently established that human infants convalescing from *H. influenzae* type b meningitis do mount an antibody response directed against cell surface-exposed outer membrane proteins of the infecting *H. influenzae* type b strain (P. A. Gulig, C. F. Frisch, G. H. McCracken, Jr., K. H. Johnston, and E. J.

Hansen, Infect. Immun., in press). These data indicate that outer membrane proteins of *H. influenzae* type b are immunogenic not only in infant rats but also in human infants and therefore may have vaccinogenic potential.

Conclusive proof that antibodies directed against *H. influenzae* type b outer membrane proteins can protect against systemic *H. influenzae* type b disease requires the use of antibodies specific for these protein antigens. The recent development of lymphocyte hybridoma technology has made possible the production of monoclonal antibodies specific for any given antigen (15). We report here the isolation of lymphocyte hybridomas producing monoclonal antibodies specific for several different *H. influenzae* type b outer membrane proteins which are known to be immunogenic in infant rats (10). We have determined that monoclonal antibodies directed against a single *H. influenzae* type b outer membrane protein can exhibit considerable heterogeneity with respect to both antigenic determinant specificity and isotype, and that a cell surface-exposed antigenic determinant of one *H. influenzae* outer membrane protein is present in a number of independently isolated strains of *H. influenzae* type b.

(A preliminary account of these findings was presented at the Annual Meeting of the American Society for Microbiology, 1 through 6 March 1981, Dallas, Tex.)

MATERIALS AND METHODS

Bacterial strains and culture media. The clinical isolate of *H. influenzae* type b employed in this study as a source of outer membrane vesicles was strain 26, which has been described previously (10). The other strains used in these experiments were blood or cerebrospinal fluid isolates obtained from George H. McCracken, Jr., Southwestern Medical School. *H. influenzae* was grown in liquid culture at 37°C in brain heart infusion broth (Difco Laboratories, Detroit, Mich.) supplemented with Levinthal base (10) as a source of hemin and NAD^+. Stock cultures of all strains were stored at −70°C in supplemented brain heart infusion broth containing 30% (vol/vol) glycerol. All cultures were harvested in the logarithmic phase of growth for experimentation.

Preparation of outer membrane vesicles. The lithium chloride extraction method of McDade and Johnston (21) was employed to prepare outer membrane vesicles from intact cells of *H. influenzae* type b. Briefly, 10 g (wet weight) of freshly harvested cells was suspended in 200 ml of LiCl extraction buffer (200 mM LiCl, 100 mM lithium acetate [pH 6.0]) in a 500-ml flask containing approximately 100 glass beads (6-mm diameter). The suspended cells were agitated vigorously on a rotary shaker at 45°C for 2 h. The cell suspension was then carefully decanted, and the glass beads were washed three times with 25 ml of LiCl extraction buffer. These washings were added to the cell suspension, which was then subjected to centrifugation at 12,000 × *g* for 15 min. The resultant supernatant was carefully collected and subjected to centrifugation at 25,000 × *g* for 15 min. The final supernatant fluid was passed over a Sepharose CL-6B column (Pharmacia Fine Chemicals, Piscataway, N.J.), and the void volume fraction, which contains the outer membrane vesicles (21), was collected and concentrated by vacuum dialysis against 50 mM Tris-hydrochloride (pH 8.0). The protein content of the vesicle preparation was determined by the method of Markwell et al. (20), and the vesicles were stored in multiple portions at −60°C until used.

H. influenzae type b outer membranes were also prepared by resolution of crude envelopes into inner and outer membrane-containing fractions by isopycnic sucrose density gradient centrifugation by the method of Hansen et al. (10).

Immunization of mice for use in hybridoma production. Several different immunization protocols were used to produce immune spleen cells for seven independent hybridization experiments. BALB/c female mice (Jackson Laboratories, Bar Harbor, Maine) (6 to 8 weeks old) were injected intraperitoneally with *H. influenzae* type b outer membrane vesicles (50 μg of protein) suspended in 0.2 ml of Freund complete adjuvant (Difco). Thirty days later, all immunized mice were given a second intraperitoneal injection with outer membrane vesicles (30 to 40 μg of protein) suspended in pH 7.2 phosphate-buffered saline (PBS). In addition, some mice were given a third intraperitoneal injection of outer membrane vesicles (30 to 40 μg of protein) 2 to 3 weeks after the second injection, and in one experiment, mice were given a fourth intravenous injection with outer membrane vesicles (25 μg of protein) 14 days after the third intraperitoneal injection. Spleens were removed from the immunized mice 3 to 4 days after their last injection of antigen for use in hybridoma production.

Construction of hybridomas. Hybridomas were produced by fusing spleen cells from immunized mice with SP2/0 plasmacytoma cells by a modification of the basic procedure of Kennett et al. (13). The SP2/0 plasmacytoma cell line, which was obtained from Roger Kennett, University of Pennsylvania Medical School, synthesizes no immunoglobulin chains (25). This cell line lacks the enzyme hypoxanthine guanine phosphoribosyl-transferase (HGPRT), is resistant to 8-azaguanine, and dies in the presence of Littlefield HAT selection medium (17). SP2/0 cells were grown in Dulbecco modified Eagle medium (DMEM) (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 15% (vol/vol) heat-inactivated fetal calf serum (HyClone FCS; Sterile Systems, Logan, Utah), 2 mM L-glutamine, and 50 U of penicillin-streptomycin (GIBCO) per ml. SP2/0 cells were grown in supplemented DMEM containing 8-azaguanine (20 μg/ml) immediately before use in hybridization experiments to ensure that no HGPRT-positive revertants were present in the cell culture.

Spleens were removed aseptically from immunized mice and teased apart gently with forceps to prepare a single cell suspension in DMEM. SP2/0 cells were harvested in the logarithmic phase of growth, and both cell types were collected by centrifugation at 270 × *g* for 10 min at 8°C and washed three times with DMEM. Total cell numbers were determined with a Coulter Counter model ZF (Coulter Electronics, Inc., Atlanta, Ga.), and viability was measured by trypan blue

exclusion. Approximately 10^8 spleen cells were mixed together with SP2/0 cells in a 50-ml conical tube at a ratio of 7 to 10 viable spleen cells per viable SP2/0 cell, and the resultant cell suspension was collected in a pellet by centrifugation at 270 × *g* for 10 min. The supernatant medium was removed, and the tube containing the cell pellet was placed in a 37°C water bath. A 0.2-ml portion of a warm (37°C) 35% (wt/vol) solution of polyethylene glycol (PEG 1000; J. T. Baker Chemical Co., Phillipsburg, N.J.) in DMEM was added to the cell pellet, which was then gently mixed with a glass rod. The cell suspension was incubated at 37°C for 3 min and was then collected by centrifugation at 270 × *g* for 4 min. Warm DMEM (5 ml) was gently dropped onto the cell pellet, which was then loosened with a glass rod. An additional 5 ml of warm DMEM was then added, and the cells were collected by centrifugation. This final cell pellet was suspended in 25 to 30 ml of DME-HY medium (13) and dispensed in 50-μl portions containing 1.6×10^5 to 5.3×10^5 cells each into microtiter plate wells (Costar Plastics, Vineland, N.J.), which were then incubated at 37°C in a humidified incubator containing a 7% CO_2 atmosphere. On day 2, a 50-μl portion of a twofold concentration of Littlefield HAT selection medium (17) was added to each well. An additional 100-μl portion of HAT was added to each well on day 3. The SP2/0 cells died in HAT within 24 to 48 h. Hybrid clones selected in HAT could usually be observed by day 6. After day 6, all wells were fed HT-glycine (13). Wells which contained growing clones were split 1:1 into two new microtiter wells; if growth continued, cells were transferred from these two wells into one well of a 24-well tissue culture plate (Costar Plastics), from which supernatants were obtained for use in screening assays to detect monoclonal antibodies directed against *H. influenzae* type b outer membrane antigens. Hybrid clones were maintained in supplemented DMEM without feeder layers.

Enzyme-linked immunosorbent assay (ELISA) procedure. Screening of hybrid clone culture supernatants for the presence of monoclonal antibodies directed against *H. influenzae* type b outer membrane antigens was performed essentially as described by Johnston (12). Outer membrane vesicles (6 μg of protein per well) employed as the source of antigen were coated onto microtiter wells in a 96-well plate (Costar Plastics) by the method of Voller et al. (27). Antigen-coated wells were washed three times with PBS containing 0.05% (vol/vol) Tween 20 (PBS-Tween). PBS-Tween (300 μl) containing 1% (wt/vol) bovine serum albumin was incubated in each microtiter well for 1 h at room temperature to saturate nonspecific protein binding sites in the plastic well. This solution was then removed by aspiration, the wells were washed three times with PBS-Tween, 100 μl of hybrid clone culture supernatant was added to the well, and the microtiter plate was incubated at 4°C overnight. Positive control wells contained mouse serum obtained from the same mouse whose spleen was employed for hybridization. Supernatant fluid was then removed by aspiration, the microtiter wells were washed three times with PBS-Tween, and alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.), prepared by the method of Voller et al. (27) and diluted 1/400 in PBS-Tween, was then added to a final volume of 200 μl in each microtiter well. The microtiter plates were incubated at room temperature for 1 h, after which the conjugated antiserum was removed by aspiration, the wells were washed three times with PBS-Tween, and 300 μl of enzyme substrate (*p*-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, Mo.]; 1 mg/ml in 10% [vol/vol] diethanolamine buffer [pH 9.8] containing 1 mM $MgCl_2$) was added to each well. Thirty minutes later, the absorbance of the solution in each well was determined spectrophotometrically at 405 nm by using a Titertek Multiscan (Flow Laboratories, McLean, Virginia). Microtiter wells in which the absorbance was at least twofold greater than background levels of absorbance obtained with antigen-free control wells were scored as positive for the presence of antibodies directed against *H. influenzae* type b outer membrane antigens.

Identification of mouse antibody isotypes. Rabbit and goat anti-mouse immunoglobulins were purchased from Cappel Laboratories, Cochranville, Pennsylvania. Affinity purified, heavy chain-specific rabbit anti-mouse subclass reagents were prepared for use as radioimmunoassay probes. These reagents were prepared from antisera obtained from rabbits immunized with the following myeloma proteins: CBPC-22 (immunoglobulin M [IgM]); TEPC 15 (IgA); MOPC 21 (IgG1); UPC 10 (IgG2a); MOPC 195 (IgG2b); and FLOPC 21 (IgG3) (Cappel Laboratories). The IgG fraction was precipitated from the antisera at 4°C with 37% (vol/vol) saturated ammonium sulfate and dialyzed against 0.01 M sodium phosphate buffer (pH 7.5). The IgG fraction was further purified by batch elution from DEAE-Sepharose (Pharmacia) after a 30-min incubation at room temperature in 0.01 M sodium phosphate buffer containing 0.05 M NaCl. The immunoglobulin fraction was then absorbed over heterologous mouse immunoglobulin-Sepharose-CL4B (Pharmacia) columns to remove anti-light chain activity and any inappropriate anti-heavy chain activity. These antibodies were then affinity purified on homologous mouse immunoglobulin-Sepharose-CL4B columns and eluted with either 0.2 M propionic acid in 0.15 M NaCl or 5 M guanidine. These purified antisubclass reagents were iodinated by a modification of the chloramine T procedure (11).

Routine antibody subtyping was performed in a solid-phase radioimmunoassay. For subtyping, goat or rabbit anti-mouse immunoglobulin (1 mg/ml, 100 μl/well) was used to coat the microtiter plates. The plates were then washed and blocked with bovine serum albumin as described above for the ELISA procedure. Culture supernatants were added and incubated for 3 h at room temperature or 37°C and then washed out. Iodinated affinity-purified rabbit anti-mouse isotype probes were added to identity the isotype of mouse antibody bound to the plate. The plates were incubated at 4°C, washed, cut, and counted in a gamma counter.

Radioimmunoprecipitation methods. (i) Method 1. Culture supernatant fluids from hybrid clones which scored positive in the ELISA test were assayed by a radioimmunoprecipitation method for the presence of monoclonal antibodies directed against *H. influenzae* type b outer membrane proteins. Outer membrane proteins in intact cells of *H. influenzae* type b were radioiodinated by the method of Hansen et al. (10). Radioiodinated cells (specific activity, 0.01 cpm per

colony-forming unit) were suspended to a final specific activity of 2×10^7 cpm/per ml of solubilization buffer (10 mM Tris-hydrochloride [pH 7.8] containing 150 mM NaCl, 10 mM EDTA, 1% [vol/vol] Triton X-100, 0.2% [wt/vol] sodium deoxycholate, and 0.1% [wt/vol] sodium dodecyl sulfate) and incubated at 37°C for 60 min. This solubilization procedure has previously been shown to effectively solubilize outer membrane proteins of *H. influenzae* type b (9). Insoluble material was removed from suspension by centrifugation at 45,000 × *g* for 1 h at 20°C; the resultant supernatant containing solubilized radioiodinated outer membrane proteins was divided into 500-μl portions containing 10^7 cpm, to which were added 500-μl portions of the hybrid clone supernatants. These mixtures were incubated for 2 h at 4°C with gentle agitation, and then 10 μg of affinity-purified rabbit anti-mouse immunoglobulin was added to each tube as a probe for mouse monoclonal antibodies attached to *H. influenzae* type b antigens. After incubation of these mixtures for 1 h at room temperature, 200 μl of a 10% (vol/vol) Formalin-treated suspension of *Staphylococcus aureus* (14) bearing protein A on its surface was added to all reaction tubes, which were then incubated at 4°C with gentle agitation for 1 h. The resultant (*S. aureus*–antibody–antigen) complexes were washed five times with solubilization buffer and processed for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiographic analysis as described previously (9, 10).

(ii) Method 2. Intact radioiodinated *H. influenzae* type b cells (2×10^7 cpm) were incubated with hybrid clone culture supernatants (500 μl) to detect monoclonal antibodies directed against cell surface-exposed antigenic determinants of *H. influenzae* type b outer membrane proteins. Radioimmunoprecipitation analysis involving intact radioiodinated *H. influenzae* type b cells as antigen was performed exactly as described previously (9), with the exception that affinity-purified rabbit anti-mouse immunoglobulin was used as a probe for mouse monoclonal antibodies, as described above.

Adsorption of hybrid clone culture supernatants with intact *H. influenzae* type b cells was accomplished by incubating 200 to 500 μl of hybrid clone culture supernatant four times sequentially with 10^{10} intact, washed *H. influenzae* type b cells for 1 h at 4°C.

Convalescent rat serum was obtained by previously published methods (10). Radioimmunoprecipitation analysis of convalescent rat sera was performed by using solubilized, radioiodinated *H. influenzae* type b cells exactly as described previously (9).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiographic identification of immunoprecipitated, radioiodinated *H. influenzae* type b outer membrane proteins were performed by previously published methods (10).

Source of chemicals and reagents. All enzymes and chemicals were obtained from Sigma unless otherwise specified. Affinity-purified rabbit anti-mouse immunoglobulin was a generous gift from Ellen Vitetta, Department of Microbiology, University of Texas Health Science Center at Dallas.

RESULTS

The source of *H. influenzae* type b outer membrane proteins employed as immunogens for hybridization experiments was outer membrane vesicles extracted from intact cells of *H. influenzae* type b strain 26 by treatment with lithium chloride extraction buffer. These outer membrane vesicles are specifically enriched in the major outer membrane proteins (Fig. 1). One of these proteins, with an apparent molecular weight of 39,000, has been shown to be at least partially exposed on the cell surface of intact *H. influenzae* type b cells and is accessible to antibody in this state (9).

Spleen cells obtained from mice immunized with *H. influenzae* type b outer membrane vesicles were fused with SP2/0 plasmacytoma cells to produce hybrid clones (lymphocyte hybridomas) which secrete monoclonal antibodies directed against *H. influenzae* type b outer membrane antigens. Seven different hybridization experiments were performed with mice immunized by the various regimens described above. Generally, between 6 and 15% of the total wells plated from each hybridization contained viable, growing hybrid clones. Screening of hybrid clone culture supernatants by ELISA for mono-

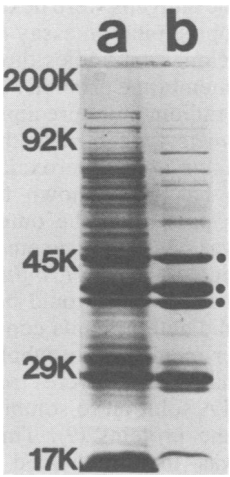

FIG. 1. Comparison of the outer membrane-containing fraction and outer membrane vesicles of *H. influenzae* type b strain 26 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. (a) The outer membrane-containing fraction was prepared by resolution of crude cell envelopes from *H. influenzae* type b into inner and outer membrane-containing fractions by the method of Hansen et al. (10); (b) the outer membrane vesicles were prepared by the LiCl extraction method described in the text. The closed circles on the right indicate the outer membrane proteins with apparent molecular weights of 45,000, 39,000, and 37,000. Coelectrophoresis of purified myosin (200,000), phosphorylase B (92,000), ovalbumin (45,000), carbonic anhydrase (29,000), and myoglobin (17,200) was used to determine the position of the molecular weight markers shown on the left. Coomassie blue staining was used to detect protein bands in the slab gel.

clonal antibodies directed against antigenic determinants present in *H. influenzae* type b outer membranes determined that approximately one-third of these hybrid clones produced antibody directed against *H. influenzae* type b outer membrane antigens. Since the frequency of hybrid production was well within the statistical limits of probable clonality by limiting dilution analysis (16) and since each anti-*H. influenzae* type b hybrid produced antibody of a single isotype, each anti-*H. influenzae* type b hybrid can be considered to be cloned. In addition, isotype analysis of the monoclonal antibodies produced by hybrid clones derived from different mouse immunization protocols revealed that the immunization regimen exerted significant effects on the isotype distribution of the resultant *H. influenzae* type b-specific monoclonal antibodies (S. M. Robertson, J. R. Kettman, and E. J. Hansen, manuscript in preparation).

Identification of monoclonal antibodies directed against *H. influenzae* type b outer membrane proteins. Culture supernatants in which the ELISA screening system detected monoclonal antibodies directed against *H. influenzae* type b outer membrane antigens were next employed in a radioimmunoprecipitation assay (method 1) to identify monoclonal antibodies directed against specific outer membrane proteins. The source of antigen in this radioimmunoprecipitation system was *H. influenzae* type b cells that had been radioiodinated by a lactoperoxidase-catalyzed system which has been shown to label only those proteins present in the outer membrane (10). Since it was possible that some monoclonal antibodies directed against *H. influenzae* type b outer membrane proteins would be specific for protein antigenic determinants concealed within the intact outer membrane of whole organisms, the radioiodinated cells were treated with a detergent-EDTA solution to solubilize all of the outer membrane proteins (9). This solubilized preparation was then incubated with hybrid clone culture supernatants containing mouse monoclonal antibodies, after which affinity-purified rabbit anti-mouse immunoglobulin was added as a specific probe for all isotypes of mouse antibody. *S. aureus* bearing protein A on its surface was next added to the reaction mixtures to precipitate the (rabbit IgG antibody–mouse antibody–*H. influenzae* type b antigen) complexes. Radioiodinated *H. influenzae* type b outer membrane proteins which were present in the resultant immune precipitates were identified by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by autoradiographic analysis (9, 10).

Seven different monoclonal antibodies obtained from five different hybridization experiments were shown to be directed against *H. influenzae* type b outer membrane proteins by means of this radioimmunoprecipitation system (Fig. 2). Three different outer membrane proteins with apparent molecular weights of 45,000, 39,000, and 37,000 were recognized by one or more of these monoclonal antibodies. These three proteins are quantitatively dominant in the LiCl-extracted outer membrane vesicles used to immunize mice for hybridoma production (Fig. 1). Monoclonal antibody 2E10 (IgG2b) was specific for an *H. influenzae* type b major outer membrane protein with an apparent molecular weight of 45,000. Monoclonal antibody 16C2 (IgG2b) was specific for an *H. influenzae* type b major outer membrane protein with an apparent molecular weight of 37,000. The other five monoclonal antibodies, which could be divided into IgG and IgM isotypes (Table 1) and which were obtained from three independent hybridizations, were all directed against an *H. influenzae* type b major outer membrane protein with an apparent molecular weight of 39,000 that has been shown to be at least partially exposed on the cell surface of *H. influenzae* type b and accessible to antibody in this state (9). It is important to note that all three of these outer membrane proteins have been shown to be immunogenic in infant rats (10) (Fig. 2).

Monoclonal antibody specific for cell surface-exposed antigenic determinants. The seven monoclonal antibodies that were shown to be

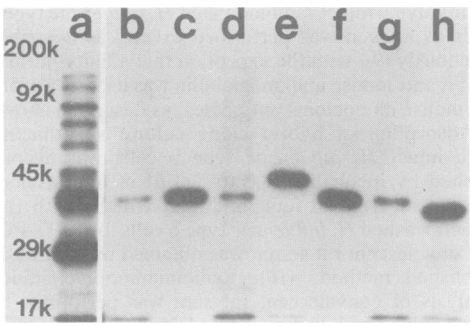

FIG. 2. Radioimmunoprecipitation of ^{125}I-labeled *H. influenzae* type b outer membrane proteins with convalescent rat sera and monoclonal antibodies. Radioiodinated *H. influenzae* cells were solubilized by treatment with solubilization buffer, and portions of the resultant solubilized outer membrane proteins containing 10^7 cpm were mixed with either 10 μl of pooled sera obtained from young rats convalescing from systemic *H. influenzae* type b disease (10) or with 500-μl portions of hybrid clone culture supernatants containing monoclonal antibodies. Radioimmunoprecipitation analysis was performed as described in the text. (a) ^{125}I-labeled outer membrane proteins precipitated by 10 μl of pooled serum from convalescent rats. ^{125}I-labeled outer membrane protein precipitated by the following monoclonal antibody: (b) 17C4, (c) 6A2, (d) 5G6, (e) 2E10, (f) 8F8, (g) 17A10 or (h) 16C2.

TABLE 1. Monoclonal antibodies directed against *H. influenzae* type b outer membrane proteins

Hybrid clone[a]	Antibody class[b]	Outer membrane protein antigen[c]
17C4	IgM	39,000
6A2	IgG3	39,000
5G6	IgM	39,000
2E10	IgG2a	45,000
8F8	IgG3	39,000
17A10	IgG2a	39,000
16C2	IgG2b	37,000

[a] Hybrid clones were derived from five different hybridization experiments. Clones 17C4 and 5G6 were produced from one experiment, 8F8 and 17A10 were from a second experiment, and the other hybrids were from three separate hybridizations.

[b] Antibody classes were determined by radioimmunoassay.

[c] Apparent molecular weight of the *H. influenzae* type b protein which is recognized by the monoclonal antibody. The *H. influenzae* type b outer membrane protein with an apparent molecular weight of 39,000 is known to be accessible to antibody on the surface of intact cells (9).

directed against *H. influenzae* type b outer membrane proteins were identified in radioimmunoprecipitation assays with solubilized *H. influenzae* type b outer membrane proteins as antigen (method 1). To determine whether any of these monoclonal antibodies were directed against cell surface-exposed portions of these outer membrane proteins, hybrid clone culture supernatants were absorbed with intact *H. influenzae* type b cells before use of these supernatants in radioimmunoprecipitation assays. Only one monoclonal antibody, 6A2, could be adsorbed out of the culture supernatants by intact cells; the remaining six monoclonal antibodies were not adsorbed by intact cells (Fig. 3). Therefore, these latter six monoclonal antibodies are apparently directed at protein antigenic determinants which are either not exposed or not accessible on the cell surface of intact *H. influenzae* type b cells.

Confirmation that the protein antigenic determinant recognized by monoclonal antibody 6A2 is exposed on the surface of intact *H. influenzae* type b cells was obtained by using 6A2 antibody in a radioimmunoprecipitation assay with intact, radioiodinated *H. influenzae* type b cells (method 2). This radioimmunoprecipitation system, which involves incubation of whole cells with antibody before solubilization of the cells, has been previously shown to be specific for antibody-accessible proteins exposed on the *H. influenzae* type b cell surface (9). When 6A2 antibody was employed in this latter radioimmunoprecipitation system, the same major outer membrane protein (39,000 molecular weight) immunoprecipitated by this antibody with presolubilized *H. influenzae* type b cells was found in the resultant immune precipitate (Fig. 3), indicating that 6A2 antibody does recognize and bind to a cell surface-exposed antigenic determinant of this protein. In contrast, 5G6 monoclonal antibody, which adsorption experiments indicated did not recognize a cell surface-exposed antigenic determinant of this same protein, did not precipitate this protein when used in this radioimmunoprecipitation system with intact, radioiodinated *H. influenzae* type b cells (Fig. 3).

Strain distribution of the antigenic determinant recognized by monoclonal antibody 6A2. It was of interest to determine whether the antigenic determinant recognized by monoclonal antibody 6A2 was unique to the immunizing *H. influenzae* type b strain or whether this antigenic determinant might be found in other strains. Accordingly, six different clinical isolates of *H. influenzae* type b collected over a 2-year period were

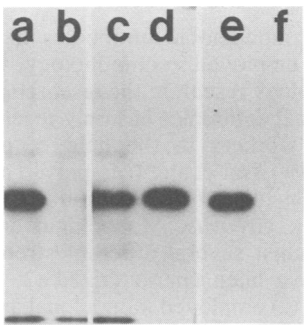

FIG. 3. Specificity of monoclonal antibody 6A2 for a cell surface-exposed protein antigenic determinant. Hybrid clone culture supernatants (500 μl each) were used as the source of monoclonal antibodies 6A2 and 5G6. Preadsorption of monoclonal antibodies with intact *H. influenzae* type b cells and radioimmunoprecipitation analysis were performed as described in the text. (a) ^{125}I-labeled *H. influenzae* type b outer membrane protein precipitated when monoclonal antibody 6A2 was incubated with solubilized *H. influenzae* type b outer membrane proteins; (b) ^{125}I-labeled outer membrane protein precipitated when monoclonal antibody 6A2 was preadsorbed with intact cells before incubation with solubilized outer membrane proteins; (c) ^{125}I-labeled outer membrane protein precipitated when monoclonal antibody 5G6 was incubated with solubilized outer membrane proteins; (d) ^{125}I-labeled outer membrane protein precipitated when monoclonal antibody 5G6 was preadsorbed with intact cells before incubation with solubilized outer membrane proteins; (e) ^{125}I-labeled outer membrane protein precipitated when monoclonal antibody 6A2 was incubated with intact, radioiodinated cells; (f) ^{125}I-labeled outer membrane protein precipitated when monoclonal antibody 5G6 was incubated with intact, radioiodinated cells.

examined for the presence of the antigenic determinant recognized by monoclonal antibody 6A2. Small (200-μl) portions of 6A2 culture supernatant were adsorbed twice with 10^{10} intact cells of each strain, and the resultant preadsorbed 6A2 culture supernatants were employed in the standard ELISA assay used to detect monoclonal antibody activity directed against *H. influenzae* type b outer membrane proteins. Intact cells of four of the six strains adsorbed 6A2 monoclonal antibody to the same extent as strain 26 (Table 2). Therefore, the cell surface-exposed protein antigenic determinant recognized by this monoclonal antibody is not unique to strain 26 and is present in these four other strains. These four *H. influenzae* type b strains have been found to differ from both strain 26 and each other by one or more proteins present in their outer membrane (E. J. Hansen, C. F. Frisch, and P. A. Gulig, unpublished data). Therefore, different *H. influenzae* type b strains can possess common or antigenically cross-reactive outer membrane proteins.

TABLE 2. ELISA values of 6A2 monoclonal antibody adsorbed with intact cells of different *H. influenzae* type b strains before testing against outer membrane vesicles of *H. influenzae* type b strain 26

Treatment of hybrid clone culture supernatant	ELISA value[a]
6A2 (unadsorbed)[b]	0.951
6A2 adsorbed with strain 26[c]	0.139
6A2 adsorbed with strain TUR	0.144
6A2 adsorbed with strain HUG	0.858
6A2 adsorbed with strain TRA	0.843
6A2 adsorbed with strain PIP	0.143
6A2 adsorbed with strain SAF	0.145
6A2 (unadsorbed) incubated in antigen-free well[d]	0.135

[a] Optical density at 405 nm after 15 min of incubation.

[b] A sample (200 μl) of 6A2 hybrid clone culture supernatant was employed as positive control.

[c] Samples (200 μl) of 6A2 hybrid clone culture supernatant were adsorbed twice with 10^{10} cells of each strain before use in the ELISA system.

[d] A sample (200 μl) of 6A2 hybrid clone culture supernatant was incubated in a well lacking strain 26 outer membrane vesicles. This well was employed as a negative control.

DISCUSSION

Although monoclonal antibodies have been extensively employed in immunology, virology, and parasitology research, the research potential of monoclonal antibodies has only recently been tapped by workers in the field of microbial pathogenesis. Cisar et al. (4) employed monoclonal antibodies to study the lectin-like fimbriae of *Actinomyces viscosus*. Monoclonal antibodies directed against several different streptococcal antigens have been characterized (2, 24), and Briles et al. (2) employed monoclonal antibodies directed against phosphocholine to prove that antibodies specific for this streptococcal cell wall antigen could protect against experimental disease caused by the encapsulated pathogen *Streptococcus pneumoniae*. More recently, two different laboratories have reported the isolation of monoclonal antibodies specific for cell surface antigens of *Neisseria gonorrhoeae* (23; K. H. Johnston, E. E. Coffee, J. R. Kettman, and S. M. Robertson, Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, B86, p. 29).

Mice immunized with *H. influenzae* type b outer membranes responded with the production of antibody-producing spleen cells that were fused with plasmacytoma cells to yield lymphocyte hybridomas secreting monoclonal antibodies directed against *H. influenzae* type b outer membrane antigens. The outer membrane-specific monoclonal antibodies obtained from these experiments undoubtedly include antibodies with individual specificities for the numerous lipopolysaccharide, lipid, and protein antigenic determinants found in the outer membrane. The use of a sensitive radioimmunoprecipitation system which can detect antibodies directed against *H. influenzae* type b outer membrane proteins permitted the identification of seven different monoclonal antibodies directed against a number of different *H. influenzae* type b outer membrane proteins (Fig. 2). The remaining monoclonal antibodies which scored positive in the ELISA system against *H. influenzae* type b outer membrane antigens, but which failed to immunoprecipitate any *H. influenzae* type b outer membrane proteins, can theoretically be divided into at least three general groups. The first group contains antibodies directed against nonproteinaceous antigenic determinants present in the outer membrane, such as lipopolysaccharide. The second group involves both monoclonal antibodies which recognize antigenic determinants present in *H. influenzae* type b outer membrane proteins, but which possess relatively low affinity for these antigens, and antibodies whose interactions with protein antigens are disrupted by the detergents involved in the radioimmunoprecipitation system. The third group of antibodies may recognize detergent-labile antigenic determinants of outer membrane proteins. Identification of those monoclonal antibodies specific for nonproteinaceous *H. influenzae* type b antigens may ultimately be accomplished by the use of ELISA systems employing purified *H. influenzae* type b lipopolysaccharide and other outer membrane components as antigens (8). Similarly, monoclo-

nal antibodies which possess the characteristics of the second group described above may eventually be shown to be specific for *H. influenzae* type b outer membrane proteins by the use of screening systems such as the protein blotting method of Towbin et al. (26).

The seven monoclonal antibodies shown to be directed against *H. influenzae* type b outer membrane proteins all recognized antigenic determinants in proteins which are known to be immunogenic in infant rats (10) (Fig. 2). Five of these monoclonal antibodies were directed against the same *H. influenzae* major outer membrane protein (39,000 molecular weight), although only one of these antibodies recognized a cell surface-exposed antigenic determinant of this protein (Fig. 3). Whether the other four monoclonal antibodies specific for this protein recognize the same or different nonexposed antigenic determinants remains to be determined. However, the immunogenicity of antigenic determinants which are apparently concealed by the outer membrane of intact *H. influenzae* type b organisms suggests that antigenic determinants buried within biological membranes may function as potent immunogens after processing by the host immune system. Alternatively, these antigenic determinants may be located on the outer membrane surface of intact *H. influenzae* type b cells in such a manner as to be inaccessible to antibody. The availability of five different monoclonal antibodies directed against two or more antigenic determinants of the same major outer membrane protein of *H. influenzae* type b will ultimately prove useful in studying the structure and function of this membrane protein. Similarly, these same monoclonal antibodies may also prove valuable in the idiotypic analysis of antibody response to cell surface structures of a pathogenic microorganism.

Finally, and most importantly, the availability of monoclonal antibodies directed against selected *H. influenzae* type b outer membrane proteins will facilitate studies on the vaccinogenic potential of these proteins. Previous work from this laboratory has established that a number of *H. influenzae* type b outer membrane proteins, in their native state in intact cells, are accessible to antibody (9). One of these outer membrane proteins, with an apparent molecular weight of 39,000, is recognized by monoclonal antibodies 8F8, 5G6, 17C4, 17A10, and 6A2. The specificity of monoclonal antibody 6A2 for a cell surface-exposed antigenic determinant of this protein will permit the use of this IgG monoclonal antibody in protection tests involving the infant rat model system, which may determine whether antibody directed against an *H. influenzae* type b cell surface-exposed protein can protect against experimental *H. influenzae* type b disease. In addition, any one of these monoclonal antibodies specific for this outer membrane protein, which is at least partially exposed on the cell surface, can be used for the affinity chromatography-based purification of this protein. Thus, with monoclonal antibody technology, it is possible to start with a complex mixture of antigens in the form of *H. influenzae* type b outer membrane vesicles and develop an antibody specific for a desired immunogen which can then be readily obtained in pure form by a single-step purification procedure. The resultant purified outer membrane protein can then be used as a vaccinogen in the infant rat model system to determine the ability of this immunogenic outer membrane protein to induce the synthesis of protective antibodies in very young mammals. These experiments are currently in progress in this laboratory.

Development of a proteinaceous vaccine to protect against systemic *H. influenzae* type b disease requires that the component protein(s) be common to, or antigenically cross-reactive with, most if not all *H. influenzae* type b strains. Previous studies have shown marked similarities and differences in the outer membrane protein content of clinical isolates of *H. influenzae* type b (1, 18), but nothing was known about the degree of antigenic relatedness of these proteins. The fact that the antigenic determinant recognized by monoclonal antibody 6A2 is not unique to a single strain of *H. influenzae* type b indicates that different strains may share one or more protein antigens (Table 2). This finding strengthens the possibility of identifying cell surface-exposed outer membrane proteins which are common to, or antigenically cross-reactive with, most or all strains of *H. influenzae* type b and which would be ideal candidates for *H. influenzae* type b vaccinogens.

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